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EVOLUTIONARY RELATIONSHIPS OF MHC GENES

Cause and effect in global climate change
Cloning the homeotic plant gene *agamous*

CHROMATOGRAPHY/HPLC
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which control the duration of effector activation, and hence the integrated intensity of cellular responses. The recent demonstration that a mutated $G\alpha$ with reduced GTPase activity is involved in cellular transformation²⁴ stresses the importance of an accurate knowledge of the true lifetime of $G\alpha$ -GTP. □

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Development of venous occlusions in mice transgenic for the plasminogen activator inhibitor-1 gene

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THE fibrinolytic potential of the vasculature is modulated primarily by the availability and activity of plasminogen activators, which convert the zymogen plasminogen into the active fibrin-degrading enzyme plasmin¹. The activities of these key regulatory enzymes are directly neutralized by their primary endogenous inhibitor, plasminogen activator inhibitor-1 (PAI-1)²⁻⁶. Although some individuals with a tendency to develop thrombotic disorders exhibit elevated levels of PAI-1 in their plasma⁷⁻¹⁰, the cause-and-effect relationship between increased PAI-1 and thrombosis is still unclear. Specifically, it is not known whether chronic depression of fibrinolytic activity results in the development of thrombosis. To address this question we developed transgenic mice in which the contribution of PAI-1 to thrombus formation could be evaluated. The results presented in this report indicate that elevated levels of PAI-1 contribute to the development of venous but not arterial occlusions.

The DNA construct used in the production of PAI-1 transgenic mice contained the murine metallothionein I promoter, human endothelial cell PAI-1 complementary DNA, and the bovine growth hormone polyadenylation signal sequence. Mice putatively transgenic for human PAI-1 showed a single, early indication of disturbance of the haemostatic system (Fig. 1a and b). A distinct area of subcutaneous haemorrhage was

apparent at the tip of the tails of mice at about 3 days after birth. This change became increasingly severe as the mice aged, until by ~12 days after birth the tip of the tail was necrotic and in addition, the mice had swollen hind feet (Fig. 1c, right). At this age, no other changes were noted. Putative nontransgenic litter mates had normal tails and hind feet (Fig. 1c, left). The necrotic tails and swollen hind feet seemed to be the result of venous occlusions detected histologically (Fig. 1e and f). These occlusions were seen in pups as young as 3 days (the earliest histological examination) and were localized exclusively in the venous circulation; the arteries of the distal tail and limbs were patent. This localization is probably related to the observed haemorrhage and oedema. The thrombi consisted predominantly of mononuclear leukocytes, but also contained platelets, erythrocytes and fibrin. The presence of muscle degeneration and inflammation in 3-day-old mice indicates very early onset of the occlusive disorder, and may at least partially explain why cellular, as opposed to simply fibrin-platelet thrombi were primarily detected. By ~2 weeks of age, the necrotic portion of the tails had completely sloughed off, whereas the swollen hind limbs appeared normal. This resolution of the occlusions in, and recovery of, the hind limbs of the mice may have been caused by compensatory mechanisms (for example, increased plasminogen activator expression) or by a decline in PAI-1 transgene expression. Therefore, the relationship between the time-dependent existence of the lesions and the levels of PAI-1 DNA, messenger RNA and protein was examined.

DNA extracted from the tails of mice displaying the circulatory disturbances described hybridized with a radiolabelled human PAI-1 cDNA probe, whereas DNA obtained from litter mates having normal tails or from control mice showed little or no hybridization (Fig. 2). Based on these results, 13 founder (G_0) animals were identified, having gene copy numbers of 1-10. In all instances, the existence of the venous occlusions correlated with the presence of the PAI-1 transgene. Assays for PAI-1 protein, activity, and mRNA were then used to correlate PAI-1

TABLE 1 PAI-1 DNA, mRNA and protein in tissues of transgenic mice

Phenotype		Tissue					
(a)		Spleen	Liver	Kidney	Heart	Brain	Lung
		ND	6.1	ND	0.8	0.1	0.1
	Control	5.8	6.9	8.0	1.1	1.1	0.2
	Normal tail	438.2	176.3	126.5	12.7	12.2	7.0
	Short tail						
(b)	Age (days):	DNA		mRNA		Protein	
		4	7	28	4	7	28
	Control	0	0	0	<0.5	<0.5	10
	Normal tail	4	2	2	1.4	2.5	<0.5
	Short tail	11	8	8	5.6	3.5	<0.5
							210
							98
							21

a. Levels of PAI-1 protein in tissues obtained from 4-day-old control and G_1 and G_2 mice of differing phenotypes. Values are expressed as nanograms of PAI-1 per g wet weight of tissue. ND, not detectable. b. PAI-1 DNA copy number and levels of PAI-1 mRNA and protein in samples of liver tissue obtained from control and G_1 and G_2 transgenic mice of various ages. Messenger RNA values are expressed as pg per μ l of total RNA and protein values are expressed as ng per g wet weight of tissue. Three to five animals were analysed in each group. DNA was prepared as described in Fig. 2. The DNA samples were then assayed by Southern blot analysis in which 10 μ g was digested with restriction enzymes, electrophoresed in a 1.0% agarose gel, blotted onto nitrocellulose, and probed with radiolabelled PAI-1 cDNA. PAI-1 gene copy-number of the transgenic mice was determined using standard amounts of the injected transgene in parallel lanes of a Southern blot of the transgenic mouse genomic DNA. RNA was prepared as described in ref. 16 and measured spectrophotometrically. RNA preparations (10 μ g) were denatured in 12 \times SSC and 15% formaldehyde at 60 °C for 15 min. The denatured RNA was applied to nitrocellulose using a slot blot apparatus (Schleicher & Schuell) and baked at 80 °C *in vacuo*. The RNA samples were then probed with radiolabelled PAI-1 cDNA, and PAI-1 mRNA levels were determined by comparison to a PAI-1 mRNA standard curve generated from the PAI-1 cDNA in pBS using the T3 promoter. For PAI-1 protein determinations, tissues were weighed and then extracted in buffer containing 0.1% Triton X-100. After centrifugation of the extracts, the supernatant fluids were analysed for PAI-1 protein using a monoclonal antibody enzyme-linked immunosorbent assay (American Diagnostica).

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FIG. 1 Morphology and histological analysis of PAI-1 transgenic mice. **a**, Putative PAI-1 transgenic mouse photographed 3 days after birth. **b**, Higher magnification view of the tail region shown in **a**. **c**, Mice photographed 12 days after birth. Left, mouse with normal tail and hind feet; right, transgenic mouse showing necrosis and sloughing of the tail and swollen hind feet. **d**, Histological cross section through the tail vein of 7-day-old PAI-1 transgenic mouse with necrosis of the tail. Note that the cellular thrombus appears to be surrounding a valve leaflet. **e**, Section through the swollen hind foot of a PAI-1 transgenic mouse. Note that the vein (V) appears to be completely occluded, whereas the artery (A) appears normal.

METHODS. The transgene construct for PAI-1 was assembled as a four-part ligation. A *Bam*HI fragment (1,397 base pairs (bp)) was isolated from a PAI-1 plasmid containing the complete coding region, 80 bases of the 5' untranslated region, and 140 bases of the 3' untranslated sequence (obtained from human umbilical vein endothelial cells). The PAI fragment was ligated on the 5' end to a 1,800-bp *Eco*RI-*Bgl*III metallothionein promoter fragment derived from pDBPV-MMT neo (342-12) (obtained from P. Howley, NIH) and on the 3' end to a 220-bp *Bam*HI-*Sph*I fragment from the polyadenylation signal sequence of bGH (obtained from F. Rottman, Case Western). The vector was pBS (Bluescribe; Stratagene) prepared as an *Eco*RI-*Sph*I fragment. The metallothionein promoter and the bGH polyadenylation signal DNA were phosphorylated and ligated to the PAI DNA overnight 12 °C at a 2:1 molar ratio. In collaboration with DNX (Athens, Ohio) eggs from B6/SJL-F₁J mice were microinjected with the ~3,400-bp *Eco*RI-*Sph*I DNA fragment and implanted in foster mothers according to established protocols. Mice were photographed with a Nikkormat camera using a medical lens and Kodak 100 ASA, daylight-balanced film. For histological evaluation, 30 G₁ and G₂ mice were killed at various ages by CO₂ inhalation. Tissues were fixed by immersion in 10% neutral-buffered formalin, processed, and embedded in paraffin according to established procedures. Tissue cross sections (5 µm) were cut, stained with haematoxylin and eosin, and examined by light microscopy.

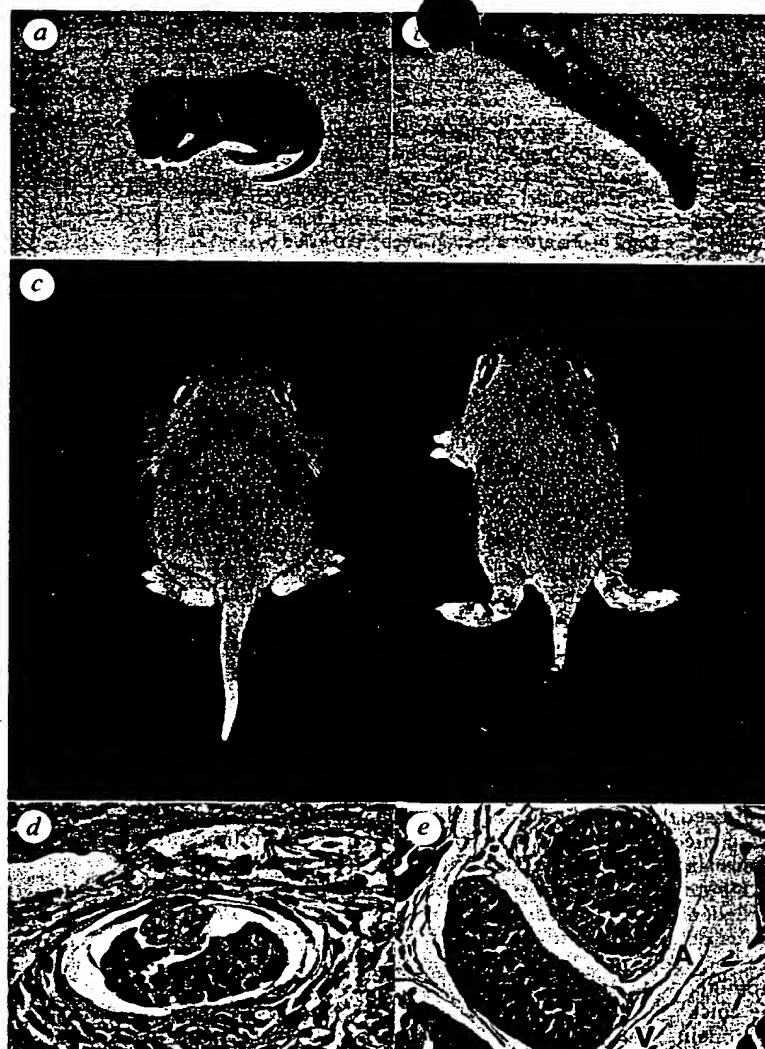
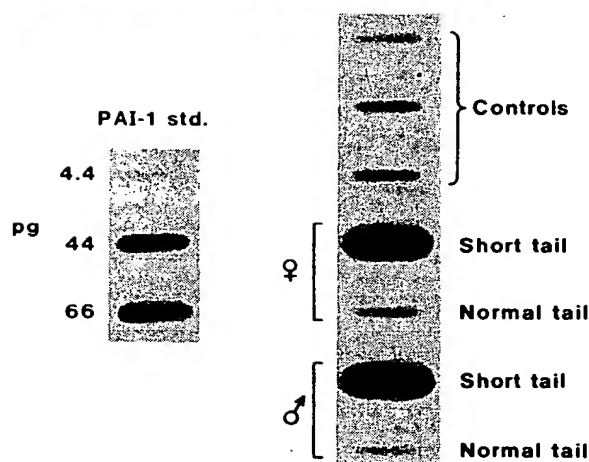


FIG. 2 PAI-1 DNA in tails of adult transgenic mice. Left, PAI DNA standard (std.); top right, tail DNA obtained from control mice; bottom right, tail DNA from female and male short-tailed and normal-tailed mice.

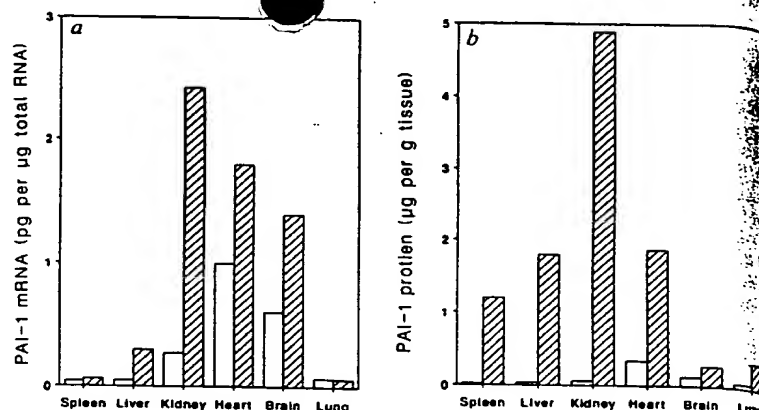
METHODS. DNA was prepared from tails by dicing them with a razor blade into 700 µl of SSTE buffer (100 mM NaCl, 1% SDS, 50 mM Tris buffer and 15 mM EDTA, pH 8.0), then digestion with 0.5 µg ml⁻¹ proteinase K at 55 °C for 12 h. The mixture was then treated with 0.2 µg µl⁻¹ RNase at 37 °C for 1 h. An equal volume of phenol was added and the mixture was extracted twice. The aqueous phase was extracted with chloroform-isoamyl alcohol (24:1) and precipitated with isopropanol. The DNA was pelleted and washed with 70% ethanol and dried under vacuum. Buffer (100 µl TE) was added to each sample and the DNA quantitated spectrophotometrically. For slot blots, 10 µg purified DNA was fixed onto nitrocellulose and probed with a radiolabelled PAI-1 cDNA.



expression with the morphological and histological profiles of the mice. Of the thirteen G₀ mice, six were selected for generation of the G₁ and G₂ lines used for subsequent analyses. Table 1(a) clearly indicates that tissue levels of PAI-1 protein also correlated directly with the presence of occlusions. Presumably, baseline PAI-1 detected in control and normal-tail animals reflects a low level of cross-reactivity between the anti-human PAI-1 monoclonal antibodies and endogenous murine PAI-1. Increased amounts of PAI-1 in the liver and kidney were expected on the basis of previous studies using the metallothionein promoter¹¹; however, the raised level of PAI-1 protein detected in spleen tissue obtained from transgenic animals was surprising,

and is as yet unexplained. The PAI-1 content of the cellular occlusions was not examined. These data show a strong link between the existence of venous occlusions and the presence and expression of the PAI-1 transgene. This relationship was examined more closely by determining the PAI-1 DNA copy-number and by measuring the PAI-1 mRNA and protein levels

FIG. 3 Induction of PAI-1 expression in 28-day-old transgenic mice with $ZnSO_4$. a, PAI-1 mRNA in tissues of transgenic mice. b, PAI-1 protein in tissues of transgenic mice. Open bars, PBS control; hatched bars, $ZnSO_4$ -injected. PBS or $ZnSO_4$ was administered to 28-day-old mice IP (10 mg kg^{-1}). Eight hours after injection, mice were killed by cervical dislocation and tissues removed for RNA and protein analyses as described in Table 1.



in the livers of mice killed at different times after birth (Table 1(b)). As expected, DNA copy-number did not vary significantly within a phenotype. Both PAI-1 mRNA and protein declined, however, in the transgenic ('short tail') mice 4–28 days after birth to levels detected in control and normal-tail (non-expressing transgenic) mice. This decline paralleled the resolution of the venous occlusions in the hind limbs, and provides additional evidence of a strict correlation between the presence and expression of the PAI-1 transgene and the development of occlusions.

The decline of PAI-1 expression during the first 4 weeks after birth could result from the loss of responsiveness of the metallothionein promoter to endogenous control mechanisms. We addressed this issue by administering $ZnSO_4$ to 28-day-old transgenic mice that had shown tail necrosis and swollen hind limbs earlier in life. This treatment induced the expression of the PAI-1 gene from baseline levels to levels that in certain tissues exceeded those observed at day 4 (Fig. 3). Plasma PAI-1 was increased by ~20-fold after $ZnSO_4$ treatment (data not shown). We conclude from these experiments that the metallothionein promoter was intact and responsive. Studies of the mouse¹² and the chick¹³ have shown that expression of endogenous metallothionein peaks shortly before birth (mouse) or after birth (chick) and declines thereafter. Taken together, these data indicate that transgenic PAI-1 may decline after day 4 as a consequence of the normal function of mechanisms governing the expression of endogenous metallothionein.

We found that the level of PAI-1 protein directly reflects the amount of PA neutralizing activity detectable (data not shown). In addition, the occlusions in the PAI-1 transgenic mice are reminiscent of those present in protein C-deficient patients in that they are localized to the venous side of the circulation¹⁴. Thus, it is conceivable that neutralization of endogenous murine activated protein C may also contribute to the observed venous occlusive disorder. Distinguishing between these and other possibilities is made particularly difficult by the fact that the critical period of evaluation is before 4 days after birth. Indeed, it has not been possible to measure the endogenous circulating levels of these murine pro-fibrinolytic factors because of the small amounts of blood obtainable from mice of this age. Alternatively, the occlusions may result from a perturbation of the vascular endothelium that is associated with chronically elevated PAI-1. In this regard, PAI-1 binds to and is protected from inactivation by vitronectin, an important component of the extracellular matrix¹⁵. The cellular nature of the venous occlusions suggests that such a perturbation may have affected the cellular adhesive properties of the venous endothelium.

Regardless of the precise mechanism, however, the results presented here provide strong evidence that elevated PAI-1 is closely associated with the development of venous occlusions. This finding has important implications in the development of

approaches to predict, treat, and possibly prevent thrombotic disorders by monitoring and regulating the endogenous fibrinolytic system.

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Drosophila Krüppel protein is a transcriptional repressor

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KRÜPPEL (*Kr*), one of the zygotically active *Drosophila* segmentation genes, is expressed in a restricted domain during the blastoderm stage of embryogenesis and is involved in the control of development of the thoracic and abdominal segments of the fly. *Kr* encodes a polypeptide containing DNA-binding zinc-finger motifs², disruptions of which yield *Kr* mutants^{3,4}. We have assayed the transcriptional activities of wild-type *Kr* protein as well as *Kr* repressor/*Kr* fusion proteins in HeLa and CV-1 cells. Wild-type *Kr* and a Lac-*Kr* chimaeric protein repressed transcription from reporter promoters in which a consensus *Kr* binding site derived from sequences within the *even-skipped* promoter⁵ had been inserted in an upstream position. We mapped the repression function of *Kr* to an alanine-rich amino-terminal region of the protein, and a Lac/*Kr* fusion protein containing only amino acids 26–110 of *Kr* repressed transcription from a reporter promoter containing

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